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Award Number: DAMD17-03-1-0352

TITLE: Is Peripheral Benzodiazepine Receptor (PBR) Gene Expression Involved in Breast Cancer Suppression by Dietary Soybean Protein?

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REPORT DATE: June 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-06-2006	Final	1 MAY 2003 - 30 APR 2006

4. TITLE AND SUBTITLE
Is Peripheral Benzodiazepine Receptor (PBR) Gene Expression Involved in Breast Cancer Suppression by Dietary Soybean Protein?

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0352

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Meharry Medical College
Nashville, TN 37208

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

14. ABSTRACT

Among many environmental factors, dietary factors play an important role in the development and progression of breast cancer. It has been established that women in Asian countries consume more soy protein than women in the United States and that the incidence of breast cancer in women in Asian countries is generally lower. While this association is correlative and no causative effect has been demonstrated, an increasing body of evidence suggests that soy protein consumption may be protective, thus reducing the risk of breast cancer development. The purpose of this study was to elucidate the molecular mechanism(s) by which dietary soy protein may offer its tumor suppressing effect. We developed a breast cancer model in female rats in which soy protein replaced casein as the dietary source of protein to investigate whether tumor development can be counteracted. The results showed a delay in the tumor formation and also a protection against the aggressiveness of the tumors in the soy protein group than in the casein group. The aggressive phenotype expression of breast cancer was correlated with the increased expression of a particular gene, peripheral benzodiazepine receptors (PBRs), implicating PBRs to be considered as a cancer promoting gene. Furthermore, the aggressive phenotype expression of breast cancer, such as increased ligand binding, increased gene expression and possible mutation(s), PBRs-mediated cholesterol transport into the nucleus, and NTPase activity of breast epithelial cells was controlled by dietary consumption of soy protein. Our studies also revealed that PBRs may play an important role in angiogenesis, and expression of some key angiogenic factors, such as b-FGF and VEGF in breast tumors was lower in soy protein group than in casein group. Therefore, it can be suggested that the breast cancer suppressing effect of dietary soy protein is mediated by inhibition of PBRs-mediated angiogenic signaling. It is thus important that this hypothesis is tested by future studies to open-up a new therapeutic approach by control of PBRs-mediated angiogenesis.

15. SUBJECT TERMS
Breast Cancer Prevention, Soybean Protein, PBRs Gene Expression

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	25	19b. TELEPHONE NUMBER *(include area code)*
U	U	U			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

The beneficial effects of dietary soybean protein in human health, particularly in breast cancer prevention, have been recently emphasized (1). However, to our knowledge, no information is available concerning the effects of dietary consumption of soybean protein on the expression of some genes, which may play a vital role in the prevention of breast cancer. It has recently been shown that ligand binding and mRNA expression of peripheral benzodiazepine receptor (PBR) is dramatically increased in the highly aggressive breast cancer cell lines and aggressive metastatic human breast tumor biopsies compared with nonaggressive cell lines and normal breast tissues (2). PBRs in aggressive breast cancer cell lines and tissue biopsies are mostly localized in and around the nucleus, which is in contrast to the largely cytoplasmic localization in nonaggressive cell lines and normal breast tissues. Furthermore, in aggressive cell lines, PBR drug ligands are found to increase the uptake of cholesterol by the nuclei and simultaneous incorporation of bromodeoxyuridine into the cells, suggesting the role of PBRs-mediated nuclear cholesterol uptake in cell proliferation (2). Numerous studies also implicate a role of nuclear cholesterol in the mechanisms underlying cell proliferation and cancer progression (2). It is not known whether the beneficial effect of dietary soybean protein on breast cancer suppression is mediated by its inhibitory effect on PBR expression, nuclear localization, and PBR-mediated cholesterol transport into the nucleus and cell proliferation. The objective of this project is to test the hypothesis that increased ligand binding, increased gene expression and possible mutation(s), and nuclear localization of PBRs, and PBRs-mediated cholesterol transport into the nucleus of breast epithelial cells are involved in cancer proliferation, and this aggressive phenotype expression can be prevented by dietary consumption of soybean protein.

WORK DONE DURING THE PROJECT PERIOD (May 1, 2003 – April 30, 2006)

APPROVED STATEMENT OF WORK

Task 1. To develop a breast cancer model by administration of DMBA to female rats fed a diet containing casein as the source of protein, and to inhibit the tumor development with soybean as the dietary protein (Months 1-18).

a. Feed weanling animals standard diets containing either casein or soybean protein and give DMBA by gavage and maintain the animals for 80 days.
b. Confirm breast cancer development in animals fed casein and suppression of breast cancer in animals fed soybean protein after 80 days of feeding.
c. Collect breast tissue for biochemical studies.
d. Develop and standardize methodologies for biochemical assays.

Task 2. To determine the role of PBRs in breast cancer suppression by dietary soybean protein (Months 18-36)

a. Maintain primary cultures of breast epithelial tissues.
b. Assay of PBR ligand binding in breast epithelial cells (Specific Aim 1).
c. Localize PBRs in cell nuclei by fluorescent microscopy (Specific Aim 2).
d. Measure nuclear uptake of cholesterol in breast epithelial cells (Specific Aim 3).
e. Measure bromodeoxyuridine uptake by breast epithelial cells (Specific Aim 4).
f. Measure ornithine decarboxylase activity of breast epithelial cells (Specific Aim 4).
g. Quantitate the expression c-fos in breast epithelial cells (Specific Aim 4).
h. Quantitate the expression of mRNA for PBRs in breast epithelial cells (Specific Aim 5).
i. Sequencing of the full-length cDNA for PBRs from breast epithelial cells (Specific Aim 6)

 Study 1. Beneficial Effects of Soy Protein in Breast Cancer Development in Female Rats

Development of Breast Tumor in Female Rats

Adult female Sprague Dawley rats were purchased at 22 days of age from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). They were housed individually in polycarbonate cages. Animals were divided into four groups. Each group contained 10 animals. Animals from groups 1 and 2 were fed with standard AIN-76A diet containing 20% casein and those of groups 3 and 4 were fed with same diet containing 20% soy protein instead of 20% casein as a form of pellet. The diets were prepared by Harlan Teklad (Madison, WI) and the composition of diets is shown in **Table 1**.

The animals were placed on the test diets at 25 days of age and remained on the diet for the rest of the study. Rats were allowed to feed and drink *ad libitum*. Mammary tumors were induced on rats of groups 2 and 4 by a single intragastric administration of dimethylbenz [a]

anthracene (DMBA) (Sigma Chemical Co, St. Louis) in sesame oil (80 mg/kg b.wt) at 50 days of age. Control animals (groups 1 and 3) received the vehicle only by gavage.

Animals were weighed and also palpated twice weekly to detect tumors beginning four weeks after the administration of carcinogen. At 122 days post-administration of DMBA, animals were killed by CO_2 asphyxiation. All tumors were weighed and measured for volume, and a section of the tumor was fixed in buffered formalin. Sections of the paraffin-embedded tumors were stained with H and E for histological analysis. The remaining tissues were used for biochemical studies.

Table 1. Composition of diets

Ingredients	Casein (g/kg)	Soybean Protein (g/kg)
Casein	200.0	-
Soy Assay Protein	-	200.0
L-Cystine	3.68	1.88
DL-Methionine	-	2.32
Sucrose	482.7273	493.586
Corn Starch	150.0	150.0
Corn Oil	50.0	44.2
Cellulose	50.0	50.0
Mineral Mix, AIN-76 (170915)	35.0	35.0
Calcium Carbonate	9.68	11.004
Cupric Carbonate	0.0057	-
Ferric Citrate	0.156	-
Sodium Bicarbonate	6.124	6.124
Vitamin Mix, AIN-76A (40077)	10.0	10.0
Choline Bitartrate	2.617	2.0
Ethoxyquin (antioxidant)	0.01	0.01

Results:

Body weights. The body weight gains were similar in all four groups. Neither DMBA administration nor the nature of dietary protein had any significant effects on the body weight gains of the animals. Data were shown in **Fig. 1**.
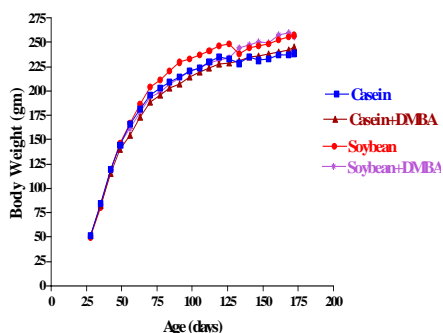

Fig. 1. Effects of DMBA injection on body weight gain of rats fed either casein or soy protein.

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Time course for tumor formation.   The time course of palpatable breast mass appearance is shown in **Fig. 2**. Replacement of casein by soy protein caused a delay in the initiation of breast tumor development. Furthermore, although multiple tumors were observed in animals of both groups, the number of tumors per rat was less in soy protein group than that in casein group at any A time period after DMBA administration.
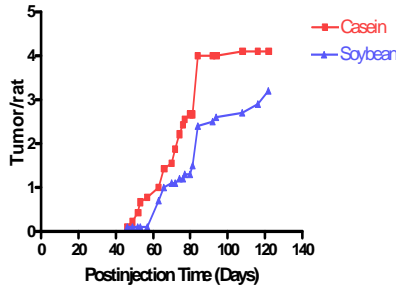

Fig.2. Time Course of Palpatable Breast Tumor

Breast tumor incidence.   Breast tumor incidence (percentage of rats with tumors) of female rats is shown in **Fig. 3**. Incidence of tumors was less in soy protein group than that in casein group.
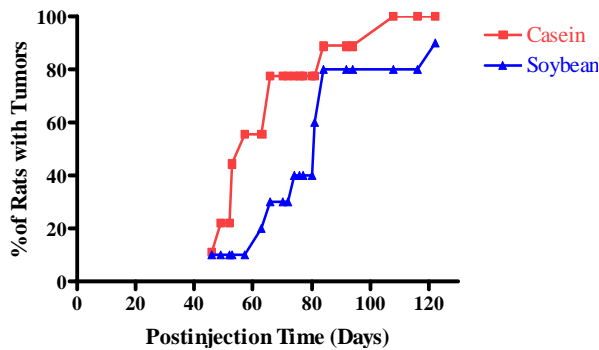

Fig. 3. Breast Tumor Incidence

Tumor characteristics.   There was no tumor in any animal, which did not receive the carcinogen regardless of whether they were fed casein or soy protein. Even though there was a difference in the time course of tumor development between the casein group and the soy protein group, the tumors were visibly apparent externally for both groups (**Fig. 4**). Some tumors in both groups had darker area, possibly implicating the cessation of angiogenesis (CAn) in that area (**Fig. 5**). However, the degree of CAn was higher in animals fed soy protein than that in animals fed casein. Further studies are needed to elucidate the mechanism by which soy protein may inhibit the progression of angiogenesis.

Although the size of the largest tumor was not significantly different between soy protein group (3.5cm x 3.3cm x 1.2cm) and the casein group (3.8cm x 2.78cm x 1.6cm), the weight of the largest tumor was 44.5% lower in soy protein group (7.52 g) than that in the casein group (13.54 g). It will be interesting to find out what is the consequence of long-term feeding of soy protein on tumor size and weight. Furthermore, even though blood vessels were visible in breast of both control groups (**Fig. 6**), dilation of blood vessels was more pronounced in the soy protein control group that that in casein control group. Thus, dilation of blood vessels may be

etiologically related to the observed cessation of angiogenesis in some sites of tumors in soy protein group.
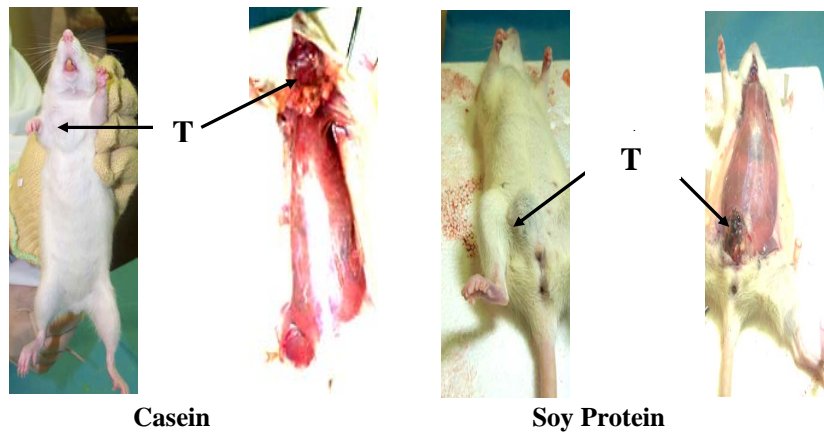

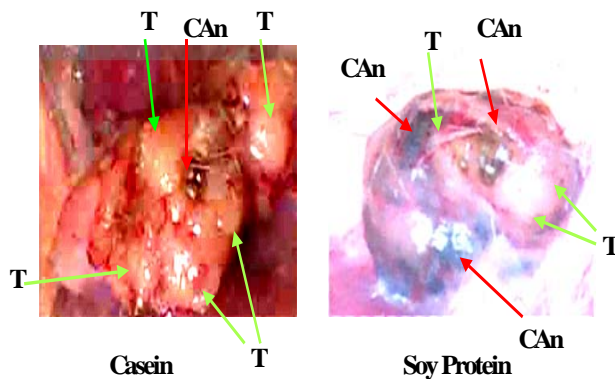**Fig. 4.** Development of Breast Tumors (T) in Animals Fed Casein and Soy Protein

Casein   Soy Protein

Fig. 5. Posible cessation of angiogenesis (Can) in breast tumors (T) in animals

Casein   Soy Protein
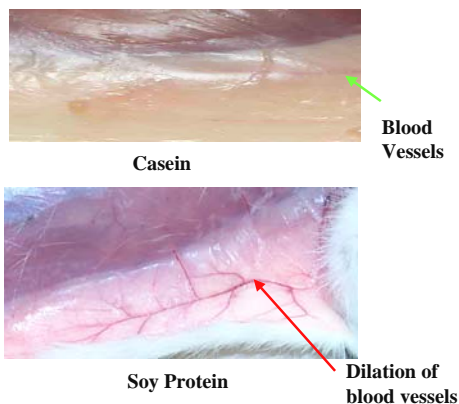
Fig. 6. Dilation of blood vessels in animals fed soy protein

Casein

Soy Protein

Pathology of breast tumors. Thirty-one specimens from animals treated with DMBA were examined microscopically. Seventeen of these specimens were from the soy protein group and 14 specimens were from the casein group. In the soy protein group, two specimens did not demonstrate a mammary gland neoplasm and 15 specimens demonstrated a grade I mammary gland adenocarcinoma. None of the specimens in this group demonstrated either grade II or

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grade III mammary gland adenocarcinoma. In the casein group, 4 specimens did not demonstrate a mammary gland neoplasm, 2 had a grade I, 6 had a grade II and 2 had a grade III mammary gland adenocarcinoma. Representative photograph of light microscopy from breast tissue of control animals regardless of whether they were fed casein or soy protein is shown in **Fig. 7**. Representative photograph of light microscopy from grade I tumor from the soy protein group is shown in **Fig. 8** and grade II and grade III tumor from the casein group are shown in **Figs. 9a** and **9b**, respectively. 100% of the mammary gland adenocarcinoma found in the soy protein group was of the non-aggressive type (grade I). However, in the casein group, a higher percentage of aggressive tumors (20% grade I, 60% grade II, 20% Grade III) were observed. Data on the histological grading of these specimens are shown in **Table 2**.
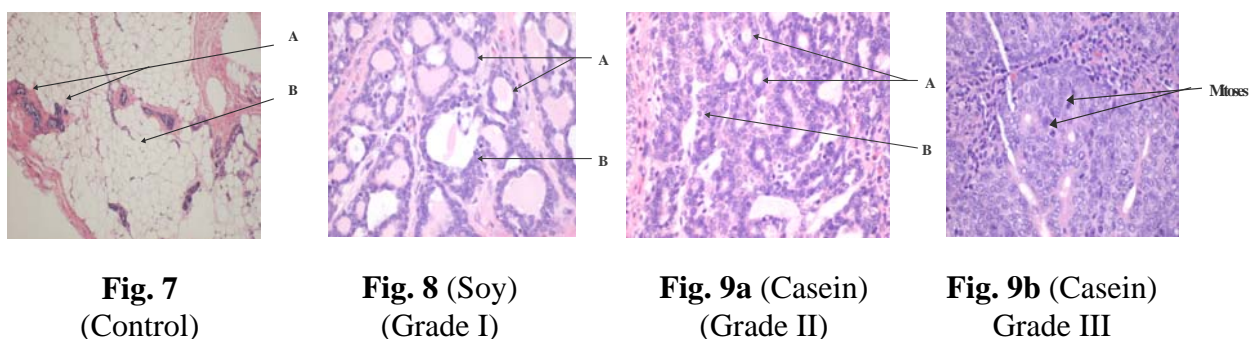

| Fig. 7 (Control) | Fig. 8 (Soy) (Grade I) | Fig. 9a (Casein) (Grade II) | Fig. 9b (Casein) Grade III |

Table 2. Histological grading of mammary gland tumors in rats-induced by DMBA

Dietary Protein	No of tumors examined	Tumor Grade			
		0	Grade I	Grade II	Grade III
Casein	14	4	2	6	2
Soy protein	17	2	15	0	0

The mitotic activity assessed in grading the tumors was verified by immunohistochemical reactions for Ki-67 antibody. Animals from the soy protein group showed less than 10% of the cells staining with Ki-67 (**Fig. 10a**). However, tumors from animals in the casein group had more than 50% of the cells staining positive with Ki-67 antibody (**Fig. 10b**). Animals from the soy protein group showed no positive cell staining with CD-31 antibody, whereas tumors from animals in casein group showed some positive staining (**Fig. 11**).

| **Figure 10a (Soy protein)** | **Figure 10b (Casein)** | **Figure 11 (Casein)** |
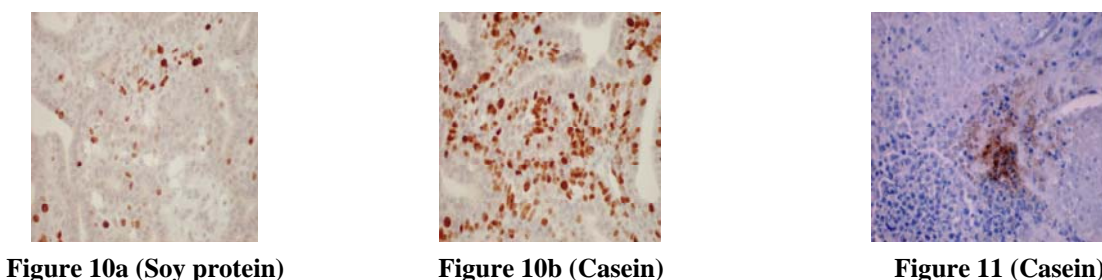
Western Blot Analysis. To check the angiogenesis, we determined the levels of two angiogenic molecular markers, VEGF and bFGF (**Fig. 12a and 12c**). We found significant differences in the levels of these markers when comparing between the two groups. In comparison to the soy protein group, casein group had 3.2- and 2-fold higher levels of VEGF and bFGF, respectively (**Figs. 12b** and **12d**). There was no significant difference between control group and soy protein group.
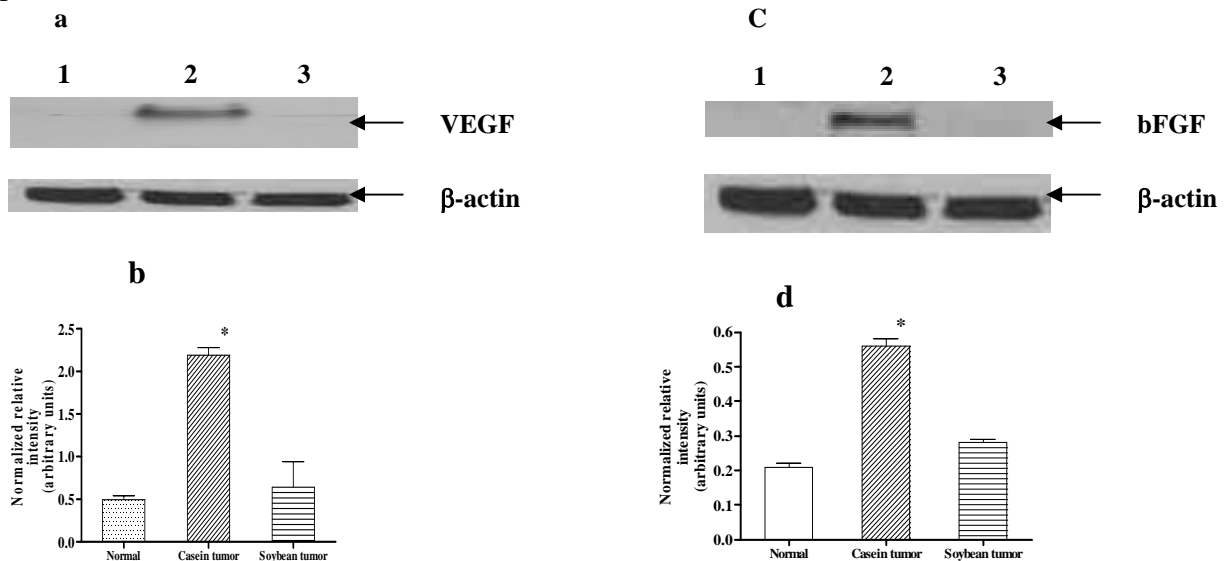

Fig. 12 (a) Western blot analysis of VEGF (n = 5) in control and DMBA-induced rat mammary gland. Lane 1- normal; lane 2 – casein; lane 3 – soy protein. (b) Histograms summarizing western blot analysis data for VEGF, *p < 0.05. (c) Western blot analysis of bFGF (n = 5) of control and DMBA-induced rat mammary gland. Lane 1- normal; lane 2 – casein; lane 3 – soy protein. (d) Histograms summarizing western blot analysis data for bFGF, *p < 0.05.

Conclusion

Although soy protein did not prevent the development of mammary tumor in animals exposed to DMBA, we found a significant and physiologically relevant delay in tumor development when soy protein was used instead of casein. Hence, this study supports a protective effect of soy protein on breast cancer development.

The histological grading was used to assess and predict the aggressiveness and clinical behavior of mammary gland adenocarcinoma [3]. This grading was based on the tubule formation within the neoplasm, nuclear pleomorphism, and mitotic count per high power field. Even though the histological grading system applied here was originally developed for human breast carcinoma, this system can be successfully applied to rat breast carcinoma. Several studies have demonstrated a similarity in histological characteristics between human and rat breast carcinoma [4, 5]. Casein group had 20% grade I, 60% grade II and 20% grade III mammary gland adenocarcinoma. However, the soy protein group had 100% grade I adenocarcinoma and no aggressive grade II or III tumor (**Table 2**). These findings suggest that the soy protein may protect against the development of a more aggressive mammary gland adenocarcinoma. Furthermore, there was a delay in the development of adenocarcinoma in the soy protein group in comparison to the animals fed casein. Recently, Simmen *et al.* [6] reported that dietary soy

protects against mammary tumorigenesis induced by direct-acting carcinogen and alters signaling pathways involving HER-2/neu and progesterone receptor.

The proliferative activity of the tumors was assessed by immunohistochemistry with Ki-67 antibody. The degree of Ki-67 staining intensity increases with aggressiveness in tumor. Positive Ki-67 correlates with the tumor's degree of differentiation, vascular invasion, and lymph node metastases. Ki-67 staining intensity was significantly reduced when casein was replaced by soy protein (Fig. 10).

While no CD-31 positive staining was observed in breast tissues of soy protein group, there was some CD-31 positive staining in casein group, suggesting that soy protein group had less angiogenesis than casein group (Fig. 11). Several lines of evidence have demonstrated that angiogenesis is essential for the growth of solid tumors and their metastases [7]. Zhou *et al*. [8] suggested that soy phytochemicals might inhibit growth of MCF-7 breast cancer cell line, an effect associated, in part, with inhibition of tumor angiogenesis. Genistein, a dominant isoflavones found in soy protein have shown anti-endothelial cell proliferation and anti-angiogenic activities [9]. The progression of breast cancer is largely affected by an imbalance that exists between angiogenic and angiostatic mediators, favoring the expression and activities of the angiogenic factors [10-15]. A pre-requisite for the treatment of breast cancer is the elucidation of the mechanisms that up-regulate the secretion of potent angiogenic factors at breast tumor sites. VEGF is well characterized as an angiogenic factor that stimulates the migration and proliferation of endothelial cells, and regulates the formation of blood vessels [16, 17]. Its elevated expression levels are correlated with aggressive breast cancer and recurrence, more so it was shown to be prognostic for overall survival and for relapse-free survival in this disease [12-15, 18]. The beneficial effect of soy protein is further evident by the presence of multiple sites of cessation of angiogenesis (Fig. 6) as well as decreased expression of angiogenic markers (VEGF and bFGF) (Fig. 12).

It has been reported that genistein has a higher binding affinity for estrogen receptor [19], as well as it is a potent inhibitor of tyrosine kinases [20]. Tyrosine kinases are essential for cell signaling networks and are regulated in normal cells. Elevated tyrosine kinase activity disturbs cellular signaling and increases malignant transformation [21]. Therefore, it is possible that dietary soy protein may offer its beneficial effect in protecting from the aggressiveness of the breast tumors by controlling the activity of tyrosine kinases.

Study 2. Increased Expression of Peripheral Benzodiazepine Receptors as Potential Markers for Breast Cancers in Rats

Development of breast tumor in female rats.   Adult female Sprague Dawley rats were purchased at 22 days of age from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). They were housed individually in polycarbonate cages. Animals were divided into two groups, group 1 and group 2. All animals were maintained on standard AIN-76A diet containing 20% casein (Harlan Teklad, Madison, WI). The animals were placed on the test diets at 25 days of age and remained on the diet for the rest of the study. Rats were allowed to feed and drink *ad libitum*. Mammary tumors were induced on rats of group 2 at 50 days of age by a single gavage administration of dimethylbenz[a]anthracene (DMBA) in sesame oil (80 mg/kg b.wt). Control animals (group1) received only the vehicle by gavage.

Animals were weighed and also palpated twice weekly to detect tumors beginning four weeks after the administration of carcinogen. At 122 days post-injection, animals were killed by

CO_2 asphyxiation. All tumors were weighed and processed for histological grading [**35**] and biochemical analysis.

Results

According to histological grading all the tumors were divided into two groups. One group had grade I (non-aggressive) mammary gland adenocarcinoma, and the second group had highly aggressive (grade II and III) mammary gland adenocarcinoma.

Binding of [3][H] Ro5-4864 to PBR in mammary gland. Differential ligand binding was observed in mammary glands. [3H] Ro5-4864 bound specifically to PBR. PBR binding was characterized by Scatchard analysis described under "materials and methods". The observed k_d values of breast tissue suggest an increase of affinity for the receptor in cancer tissues (**Fig 13** and **Table 3**). B_{max} was significantly higher in breast tumor than that in normal breast. However, in comparison to normal tissues, the PBR density was significantly higher in aggressive tumor tissues (2.8 fold) than non-aggressive tumor tissues (1.4-fold).
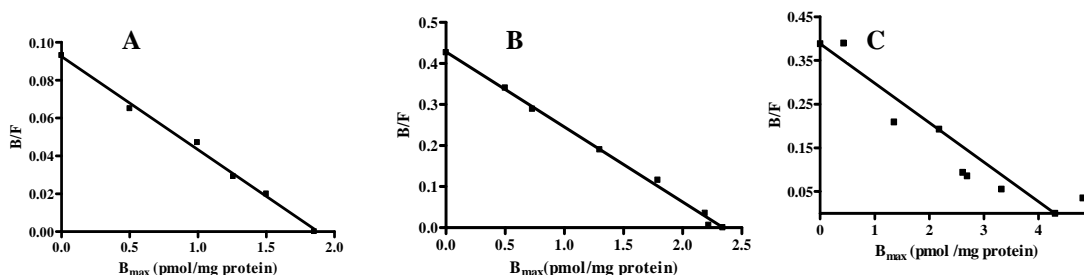

Fig. 13. Representative Scatchard Analysis of [3H] Ro5-4864 Binding to PBR in Rat Mammary Gland Membranes: A – Normal, B – Non-aggressive tumor, C – Aggressive tumor

Table 3. Binding Characteristics of PBRs in Normal and DMBA-induced Rat Mammary Gland
Results are expressed as mean ± SE of 5 different observations. *Significantly different (p<0.05)

Parameters	Normal	Cancer	
		Non-Aggressive	Aggressive
B_{max} (pmol/mg protein)	1.94 ± 0.24	2.96 ± 0.44*	4.43 ± 0.33*
K_d (nM)	42.90 ± 7.63	2.29 ± 0.25*	5.93 ± 0.98*

Cholesterol transport. Transport of cholesterol was determined as the incorporation of [3H] cholesterol in intact nucleus acquired from normal and cancer tissue. Experiments were carried out using 10μM [3H] cholesterol. Significant increase of cholesterol transport was observed in both non-aggressive and aggressive breast cancer tissue compared to normal (**Fig. 14**).
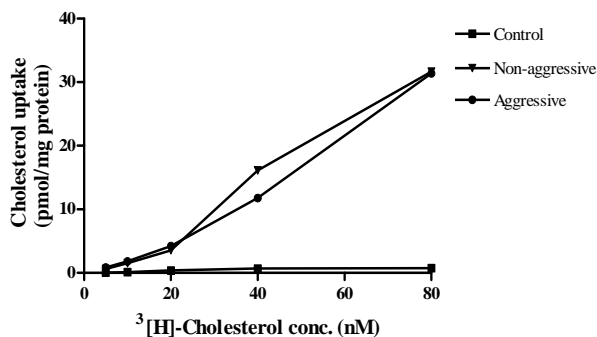

Fig. 14. Nuclear Cholesterol Transport in Normal and DMBA-induced Rat Mammary Glands. Data is representative of 5 independent experiments reported as means ± SEM. *P < 0.05.

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Binding of [3][H] Ro5-4864 to nucleus PBR in mammary gland. The observed k_d values suggest an increase of affinity for the nuclear receptor in cancer tissues (**Fig. 15 and Table 4**). B_{max} was remarkably higher in both aggressive and non-aggressive tumor tissue than in normal tissue.
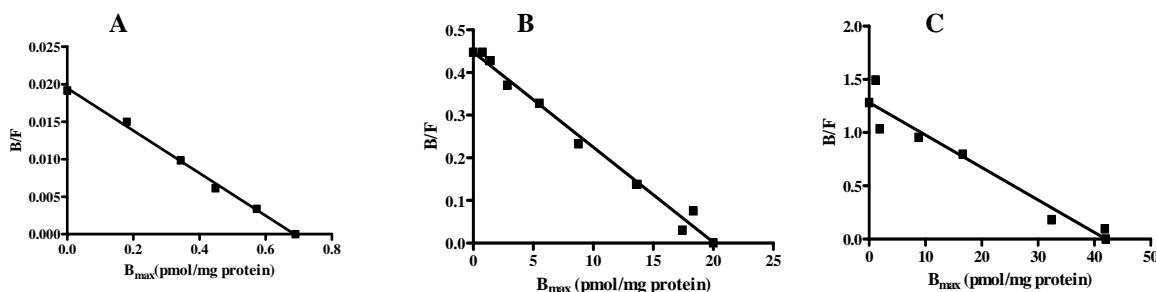

Fig. 15. Representative Scatchard Analysis of [3H] Ro5-4864 Binding to PBR in Nucleus of Rat Mammary Gland: A – Normal, B – Non-aggressive tumor, C – Aggressive tumor

Table 4. Binding Characteristics of PBRs in Nucleus of Normal and DMBA-induced Rat Mammary Gland

Parameters	Normal	Cancer	
		Non-aggressive	Aggressive
B_{max} (pmol/mg protein)	0.65 ± 0.05	21.70 ± 3.406*	44.87 ± 6.74*
K_d (nM)	8.91 ± 7.81	6.828 ± 1.887	5.56 ± 1.31

Results are expressed as mean $\pm$ SE of 5 different observations. *Significantly different (p<0.05)

NTPase activity. According to **Fig. 16**, NTPase activity was higher (130%) in aggressive cancer tissue in comparison to normal mammary gland. No significant change was observed in a non-aggressive tumor compared to normal.
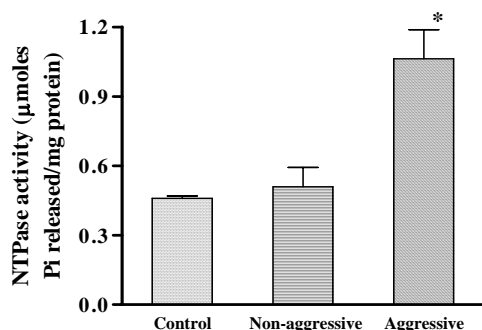

Fig. 16. Nuclear NTPase Activity in Normal and DMBA-induced Rat Mammary Glands. Results are expressed as mean $\pm$ SE of 5 different observations. *P < 0.05.

Endogenous nuclear cholesterol level. 26% increase in cholesterol level was observed in aggressive cancer tissues compared to a normal gland (**Fig. 17**). There was no significant change in cholesterol level in non-aggressive mammary gland compared to normal mammary gland.
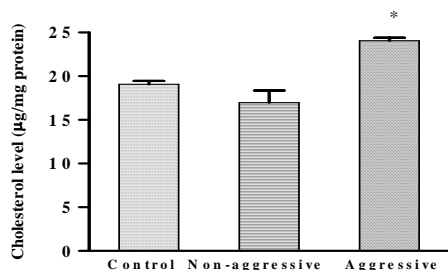

Fig. 17. Endogenous Nuclear Cholesterol Levels in Normal and DMBA-induced Rat Mammary Glands. Results are expressed as mean $\pm$ SEM of 5 different observations. *P < 0.05.

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Western blot analysis. At the level of protein regulation, we measured changes of the 18-kDa and 32-kDa subunits of PBR separately (**Fig. 18**). We found significant changes in the levels of both subunits when comparing the normal vs tumor tissues. In case of 32-kDa subunits, PBR expression was increased by 42.9% in aggressive tumor than normal mammary gland (Figure 6), whereas between control and non-aggressive tumor tissue no significant difference was observed. The expression of 18-kDa subunit of PBR was increased (45.5%) in aggressive mammary tissue in comparison to the normal. Contrary to the 32-kDa subunit, a significant decrease (31.82%) of the expression of 18-kDa subunit was observed in non-aggressive mammary gland in comparison to the control animal.
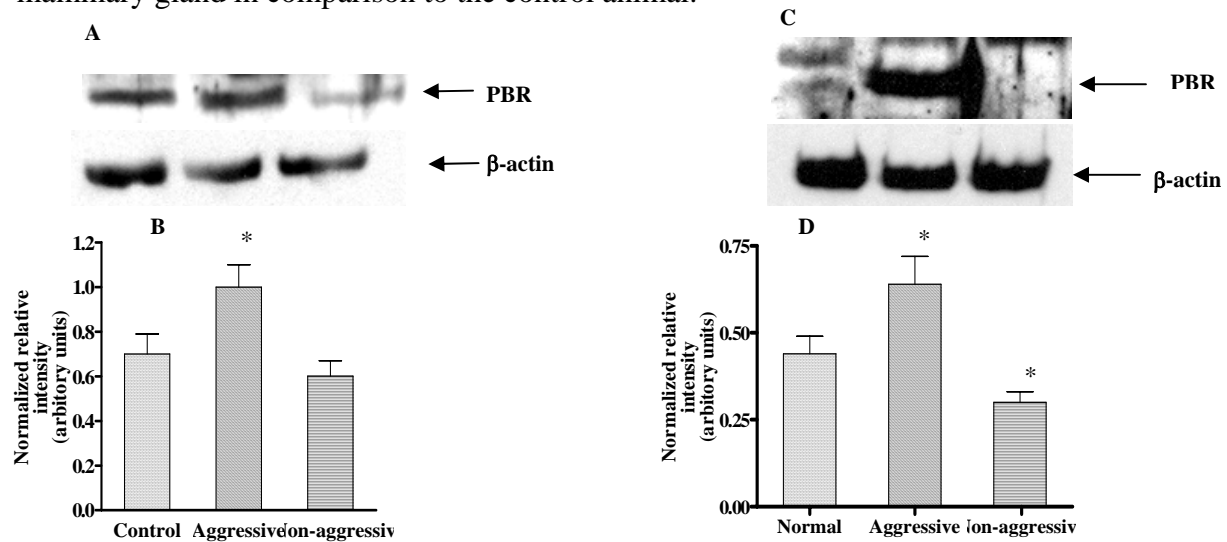

Fig. 18. (A). Western blot analysis of the 32-kDa PBR subunit in rat mammary gland (n=5). Lane 1- Normal; lane 2 – Aggressive; lane 3 – Non-aggressive. **(B)**. Histograms summarizing western blot analysis data, *P < 0.05. **(C)**. Western blot analysis of the 18-kDa PBR subunit in rat mammary gland (n = 5). Lane 1- Normal; lane 2 – Aggressive; lane 3 – Non-aggressive. **(D)**. Histograms summarizing western blot analysis data for the 18-kDa PBR subunit, *P < 0.05.

Conclusion

PBR and its endogenous ligand DBI have been detected in many benign and malignant tissues of various species. PBR and DBI have previously been detected in acinar cells of rat breast tissue and at a higher density in DMBA-induced breast tumors [22]. In our experiments, we also find an increase in the number of receptors available for binding (B_{max}) (Fig. 13, Table 3). In fact, tumor tissue shows lower k_d indicating higher affinity of ligand to these tissue. Based on these data the involvement of PBR and DBI in the regulation of function and growth of rat mammary cells may be suggested.

The physiological role of PBR is still debated. Its major function in endocrine tissues and some cell lines seems to be associated with cholesterol transport and steroidogenesis [23]. We have found an increase in the density of PBR in nucleus of breast tumors (Fig. 15 and Table 4), which may be responsible for increased transport of cholesterol into the nuclei of breast cancer tissue (both aggressive and non-aggressive) in comparison to normal tissue (Fig. 14). Even though endogenous nuclear cholesterol level was not different between normal and non-

aggressive breast tumor, there was a significantly higher level of cholesterol in nucleus of aggressive breast tumors. We do not have any explanation at this time why basal cholesterol level was also high in normal tissue. Cholesterol is a lipid found in many biological membranes. Studies have also implicated a role of nuclear cholesterol in mechanisms underlying cell proliferation and cancer progression [22, 24]. We suggest that endogenous PBR ligands bind to PBR found on the nuclear membrane and facilitate cholesterol transport into the nucleus (Fig. 14, Table 4). Cholesterol is then mobilized into the nucleus. Cholesterol's presence in the nucleus may change the dynamics of the nuclear membrane, such as fluidity, or associate itself as part of the nuclear membrane. When membrane fluidity is altered, signals that direct cell proliferation pathways indicate numerous signaling cascades in the cell [24].

Our results clearly demonstrate that the nuclear NTPase is sensitive to the cholesterol content of the nuclear membrane in aggressive breast tumors (Figs 16 & 17). Czubryt et al. [25] showed that the nuclear membrane cholesterol increased *in vivo* and the NTPase activity increased with it. The incorporation of cholesterol into the nuclear membrane in the present study may alter NTPase activity via a change in membrane rigidity. The response of cholesterol-enriched nuclei suggests that cholesterol incorporation has left the membrane integrity more susceptible to damage from stressful stimuli like cancer.

The expression of 32-kDa subunit of PBR protein increases only in aggressive tumors and not in non-aggressive tumors (Fig. 18). Furthermore, 18-kDa subunit of PBR also increases in aggressive mammary tissues whereas it is decreased in non-aggressive mammary tissues (Fig. 18). However, it should be noted that the commercially available 18kDa PBR antibody did not give a clean band in comparison to 32 kDa PBR antibody. Even though the exact mechanism is not known at this time, it may be suggested that 32-kDa proteins may be a polymorphic form of 18-kDa PBR proteins. It is known that aggressive human breast cancer cells contain mainly a PBR dimer, which increases cholesterol transport into the nucleus and cell proliferation [23]. Delavoie et al. [26] also proposed that PBR polymer might be the functional unit responsible for ligand-activated cholesterol binding, and that PBR polymerization is a dynamic process modulating the function of this receptor in cholesterol transport and other cell-specific PBR-mediated functions.

PBR nuclear localization and increase in cholesterol transport in breast cancer implicates that PBR has a role in nuclear functions. Many molecular and cellular changes are currently used as a factor in diagnosing breast cancers as prognostic indicators. Effective anticancer therapies are key in treating breast cancer. This study clearly indicates that PBR is an important molecule in cancer diagnosis and progression. Data on this study will provide a better understanding of the interplay involving PBR and other molecules, especially cholesterol, in the breast cancer signaling cascade.

Study 3. Mechanistic Link between Inhibition of the Expression of PBRs and Control of Breast Cancer Aggressiveness: Role of Soy Protein

Binding of [3][H] Ro5-4864 to PBR in mammary gland. Differential ligand binding was observed in mammary gland. [3H] Ro5-4864 bound specifically to PBR. PBR binding was characterized by Scatchard analysis. The observed k_d values of breast tissue suggest an increase of affinity for the receptor in DMBA-induced breast tumors (**Table 5**). B_{max} was significantly higher in DMBA-induced breast tumors than that in normal breast. However, dietary soy protein caused significantly lesser increase (1.6-fold) in B_{max} than casein (2.3-fold).

Table 5. Binding Characteristics of PBRs in Normal and Cancerous Rat Mammary Gland: Effects of dietary protein

Parameters	Casein	Casein + DMBA	Soybean	Soybean +DMBA
B_{max} (pmol/mg)	1.8 ± 0.5	6.6 ± 0.7*	1.8 ± 0.54	2.0 ± 0.4*
K_d (nM)	11.9 ± 3.1	3.4 ± 1.1	13.7 ± 2.5	3.5 ± 0.5

Results are expressed as mean $\pm$ SE of 3 different observations. *Significantly different (p<0.05)

Cholesterol transport. Transport of cholesterol was determined as the incorporation of [^{3}H] cholesterol in intact nucleus acquired from normal and DMBA-induced breast tumors. Experiments were carried out using 10µM [^{3}H] cholesterol. Significant increase of cholesterol transport was observed in tumors compared to normal (**Fig. 19**). No significant difference was noticed between casein-fed tumors and soybean-fed tumors.
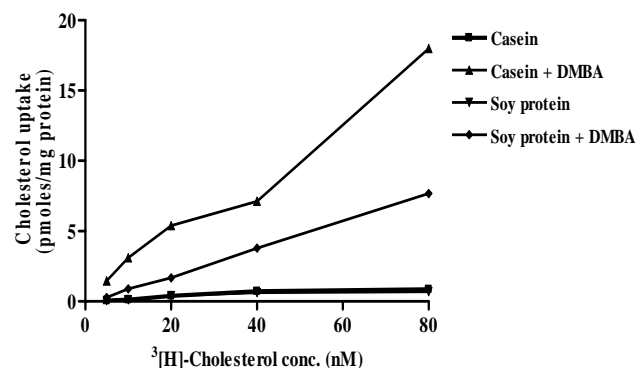

Fig. 19. Nuclear Cholesterol Transport in Normal and DMBA-induced Rat Mammary Glands. Data is representative of 3 independent experiments.

Binding of ^{3}[H] Ro5-4864 to nucleus PBR in mammary gland. The observed k_d values suggest an increase of affinity for the nuclear receptor in tumors (**Table 6**). B_{max} was remarkably higher in tumors than normal tissues. However, B_{max} was lower in soy protein-fed tumors than casein-fed tumors.

Table 6. Binding Characteristics of PBRs in Nucleus of Normal and Cancerous Rat Mammary Gland: Effects of dietary protein

Parameters	Casein		Soy protein	
	- DMBA	+ DMBA	- DMBA	+ DMBA
B_{max}	2.6 ± 0.1	37.2 ± 6.7	1.6 ± 0.3	11.0 ± 1.4*
K_d	8.9 ± 1.8	4.0 ± 1.3	10.5 ± 1.0	3.7 ± 0.8

Values are expressed as mean $\pm$ SE of 3 different observations. Nuclear PBRs density is significantly lower in soy protein group than casein group (p < 0.05)

Western blot analysis of diazepam binding inhibitor (DBI).   At the level of protein regulation, we measured changes of the DBI, the endogenous ligand of PBRs (**Fig. 20**). We found significant changes in the levels when comparing the normal vs tumor tissues. Also, DBI expression was increased in casein-fed tumor than soy protein-fed tumor tissue.
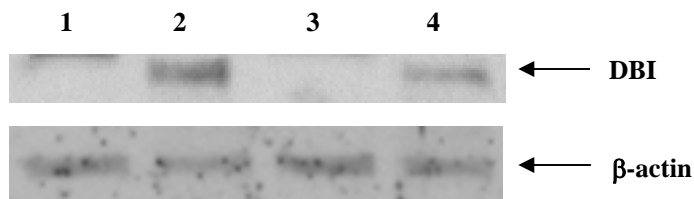
Fig. 20. Western blot of DBI and β-Actin in Breast Tissues. Lane 1: Normal (casein); Lane 2: Tumors (casein); Lane 3: Normal (soy protein); Lane 4: Tumors (soy protein)

RT-PCR of PBRs of rat mammary gland tumors. RT-PCR from both control, casein-fed and soy protein-fed tumor tissues yielded a product of ~0.47 Kb cDNA of PBR (**Fig 21**).
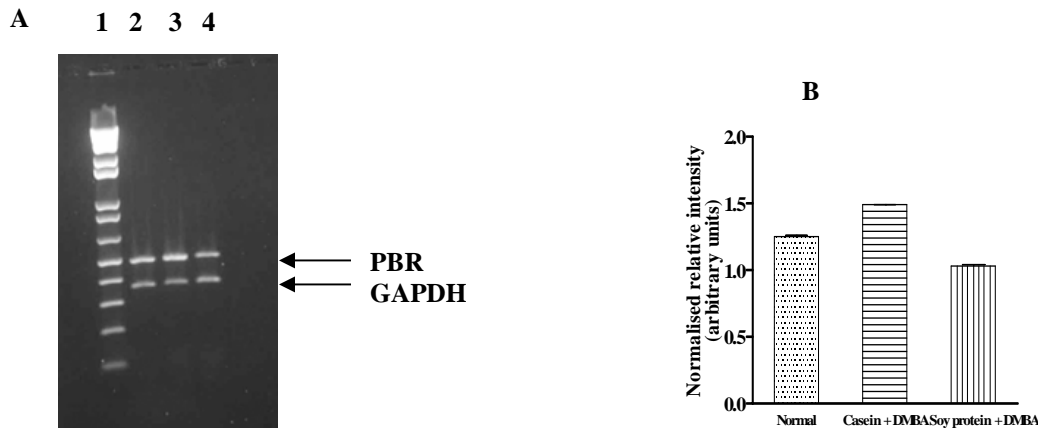

Fig. 21. RT-PCR of PBR in rat mammary gland tumors. (A) A representative gel showing 0.47 Kb product of PBR and 0.3 Kb product for GAPDH. Lane 1: 100kb plus marker; lane 2: Normal; lane 3: Casein-fed group; lane 4: Soy protein-fed group. (B) Graph showing up-regulation of PBR expression in the group of casein-fed diet normalized with GAPDH. N = 3.

Effect of soy protein on PBR gene expression. No mutation was observed in the nucleotide sequence of these cDNA fragments of PBR gene as a result of the variation of diets. However, both Northern blot (**Fig. 22**) and Real-Time PCR (**Fig. 23**) analysis revealed that replacement of casein by soy protein in the diet reduces the expression of PBRs gene by more than 60%.
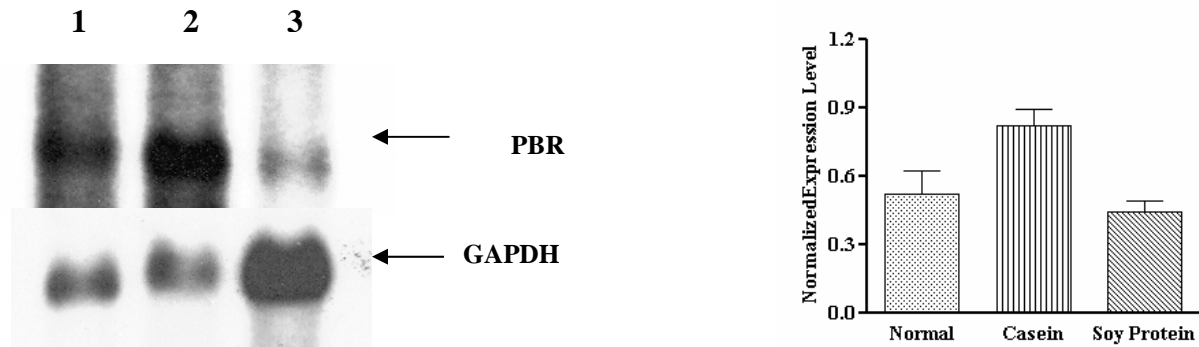

Fig. 22. Effects of diet on expression of PBR gene in normal and DMBA-induced rat mammary gland tumors. (A) One representative blot for PBR mRNA and same blot reprobed for GAPDH expression. (B) Histograms summarizing northern blot analysis data for PBR, $p \leq 0.05$, N = 3.
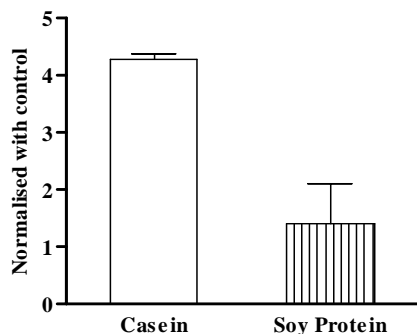
Fig. 23. Effects of diet on expression of PBR gene in DMBA-induced rat mammary gland tumors by real time RT-PCR analysis.

Immunihistochemical staining of PBRs. Tumors from animals of the soy protein group showed less cells staining with PBR antibody (**Fig. 24A**) than those from the casein group (**Fig. 24B**).
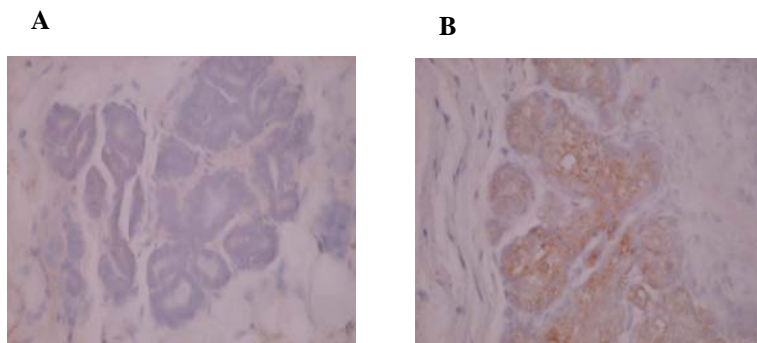
A   **B**

Fig.24. Immunohistochemical analysis of PBRs. (A) Immunohistochemical sections from breast tumors in soy protein group (40X). (B) Immunohistochemical sections from breast tumors in casein group (40X).

Conclusion

PBR and its endogenous ligand DBI have been detected in many benign and malignant tissues of various species. PBR and DBI have previously been detected in acinar cells of rat breast tissue and at a higher density, in DMBA-induced breast tumors [22]. In our experiments, we also find an increase in the number of receptors available for binding (B_{max}) (Table 5). In fact, tumor tissue shows lower k_d indicating higher affinity of ligand to these tissue. The density of the receptors was significantly reduced by replacement of casein by soy protein. Furthermore, the expression of the PBRs endogenous ligand DBI while was higher in tumors than normal tissues, replacement of casein by soy protein in the diet significantly decreased its expression (Fig.20). Based on these data the involvement of PBR and DBI in the regulation of function and growth of rat mammary cells may be suggested.

The physiological role of PBR is still debated. Its major function in endocrine tissues and some cell lines seem to be associated with cholesterol transport and steroidogenesis [23]. We have found an increase in the density of PBR in nucleus of breast tumors (Table 6), which may be responsible for increased transport of cholesterol into the nuclei of breast cancer tissue (both casein and soybean protein group) in comparison to normal tissue (Fig. 19). Cholesterol is a lipid found in many biological membranes. Studies have also implicated a role of nuclear cholesterol in mechanisms underlying cell proliferation and cancer progression [22, 24]. We suggest that endogenous PBR ligands bind to PBR found on the nuclear membrane and facilitate cholesterol transport into the nucleus (Fig. 19, Table 6). Cholesterol is then mobilized into the nucleus. Cholesterol's presence in the nucleus may change the dynamics of the nuclear membrane such as fluidity or associate itself as part of the nuclear membrane. When membrane fluidity is altered,

signals that direct cell proliferation pathways indicate numerous signaling cascades in the cell [24].

The response of cholesterol-enriched nuclei suggests that cholesterol incorporation has left the membrane integrity more susceptible to damage from stressful stimuli like cancer. It is interesting to note that NTPase activity and endogenous cholesterol level in nuclei was lower in non-aggressive tumors than aggressive tumors (Figs 16 and 17, Study 2). This may be related to the the beneficial effect of dietary consumption of soy protein in controlling the development of aggressive breast tumors.

Both Northern blot (Fig. 22) and Real-Time PCR (Fig. 23) analysis revealed that replacement of casein by soy protein in the diet reduces the expression of PBRs gene by more than 60%. PBR nuclear localization and increase in cholesterol transport in breast cancer implicates that PBR has a role in nuclear functions. Many molecular and cellular changes are currently used as a factor in diagnosing breast cancers as prognostic indicators. Effective anticancer therapies are key in treating breast cancer. This study clearly indicates that PBR is an important molecule in cancer diagnosis and progression. Data on this study will provide a better understanding of the interplay involving PBR and other molecules especially cholesterol in the breast cancer signaling cascade. Furthermore, Soybean protein appears to have the beneficial effect in breast cancer development by down-regulating the expression of PBRs as well as nuclear cholesterol uptake. Most probably, this beneficial effect of soy protein in breast cancer development is mediated by its inhibitory effect on the expression of PBRs-mediated angiogenic signaling molecules as suggested by Study 1.

Study 4. Control of mammary epithelial cell proliferation by soy protein

Western Blot Analysis of AP-1 Transcription Factor. In order to have an idea on the possible mechanism by which soy protein mediates its protective effect on the development of aggressive breast tumors, we collected data on the expression of member of the AP-1 family of transcritption facors, namely c-Fos and c-Jun which are involved in cell proliferation. Western blot analysis indicated that in comparison to casein, soy protein reduced the level of c-Fos in tumors by 31%, (**Fig. 25**). Data were normalized against β-actin as positive control.
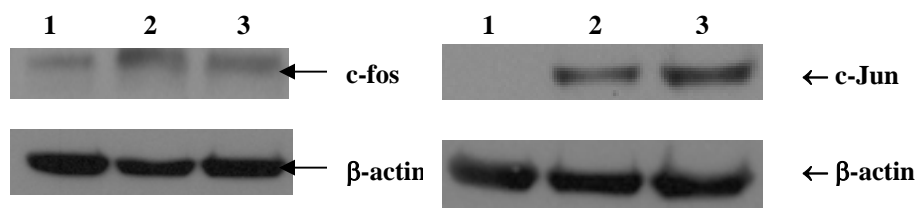

Fig 25. Western blot of c-Fos, c-Jun and β-Actin in Breast Tissues. Lane 1: Normal; Lane 2: Tumors (casein); Lane 3: Tumors (soy protein)

ELISA assay of the activity of different MAPK. According to **Fig. 26**, no significant change was observed in the activity of total ERK, whereas the activities of both p38 and phospho JNK was significantly increased in the casein tumor group compared to the soy protein tumor group.
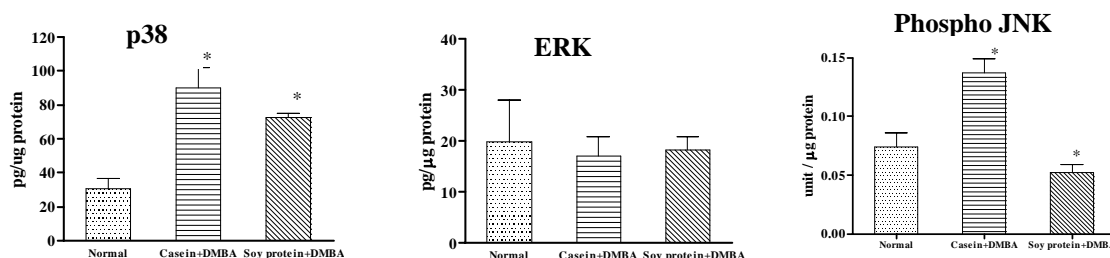

Fig. 26. Activity of different MAPK

Western Blot Analysis of JNK-2. To confirm further that replacement of casein by soy protein in the diet lowers the expression of phosphor JNK-2, we performed Western blot analysis (**Fig. 27**). Expression of JNK-2 was higher in tumors than normal breast. However, the expression of JNK-2 was significantly less in soy protein group than casein group.
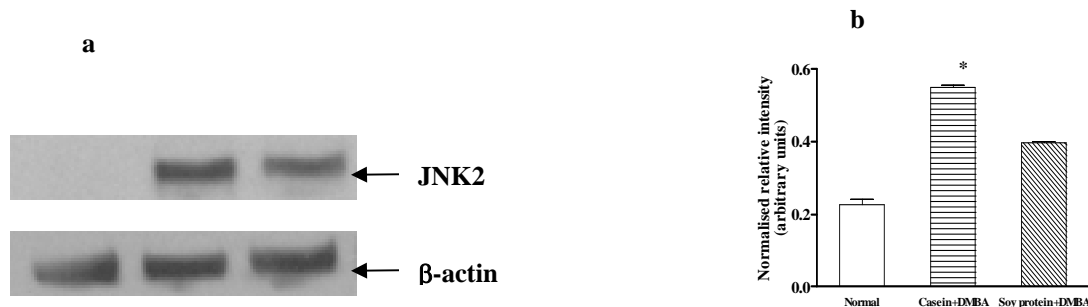

Fig.27. (a) Western blot analysis of JNK-2 (n = 3 in each group) in control and DMBA-induced rat mammary gland tumors. Lane 1- normal; lane 2 – casein; lane 3 – soy protein. (b) Histograms summarizing western blot analysis data for JNK-2, *p < 0.05.

Cell proliferation studies on amary epithelial cells. We have been successful in culturing the mammary epithelial cells from tumors from both casein and soy protein group (**Figs 28A and 28B**). However, there is a remarkable difference in the maintenance of these cells in culture depending on whether the tumors are from casein or soy protein group. While we have been successful in growing the cells from the casein group for more than 3 passages, we could not grow the cells for more than one passage for the soy protein group. This may be related to their difference in cell proliferation property (grading).
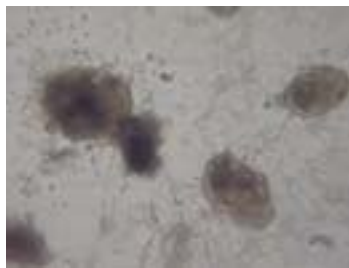

Fig. 28A (Casein)   **Fig. 28B** (Soy protein)

According to **Fig 29,** we observed that when we added different concentration of a PBR agonist Ro5-4864, cell proliferation was increased in the mammary epithelial cells derived from the casein group and not from the soy protein group. At the same time, an PBRs anatagonist, pK11195 offers an opposite effect on cell proliferation in the casein group (**Fig. 30**). This suggests that soy protein intereferes in the PBRS-induced cell proliferation in breast tumors, and thereby offers its beneficial effect in controlling breast tumor development. This study further suggests an application of a PBRs anatogonist as a potential therapeutic drug in the control of breast cancer progression.
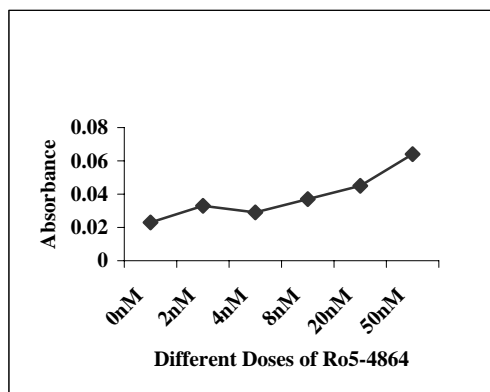
Fig. 29. Ro5-4864 induced cell proliferation of mammary epithelial cells isolated from tumor from the casein group
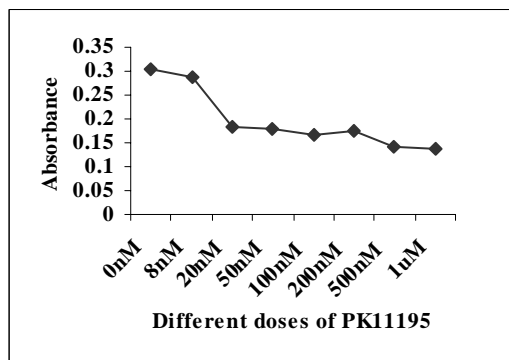

Fig. 30. PK11195 induced cell proliferation of mammary epithelial cells isolated from tumor from the casein group

Conclusion: Consumption of dietary soy protein offers its beneficial effect on breast cancer development by inhibiting cell proliferation by decreasing the expression of AP-1 transcription factors, namely c-fos and c-jun as well as several members of MAPK, such as P-38 and JNK-2. This decrease in the expression of AP-1 transcription factors and MAPK is associated with a decrease in the expression of PBRs by dietary consumption of soy protein.

Summary of the Whole Project

Among many environmental factors, dietary factors play an important role in the development and progression of breast cancer. It has been established that women in Asian countries consume more soy protein than women in the United States and that the incidence of breast cancer in women in Asian countries is generally lower. While this association is correlative and no causative effect has been demonstrated, an increasing body of evidence suggests that soy protein consumption may be protective, thus reducing the risk of breast cancer development.

The purpose of this study was to elucidate the molecular mechanism(s) by which dietary soy protein may offer its tumor suppressing effect. We developed a breast cancer model in female rats in which soy protein replaced casein as the dietary source of protein to investigate whether tumor development can be counteracted.

The results showed a delay in the tumor formation and also a protection against the aggressiveness of the tumors in the soy protein group than in the casein group. The aggressive phenotype expression of breast cancer was correlated with the increased expression of a particular gene, peripheral benzodiazepine receptors (PBRs), implicating PBRs to be considered as a cancer promoting gene. Furthermore, the aggressive phenotype expression of breast cancer, such as increased ligand binding, increased gene expression and possible mutation(s), PBRs-mediated cholesterol transport into the nucleus, NTPase activity, cell proliferation, increased expression of AP-1 transcription factors as well as some members of MAPK family (P-38 and JNK-2) of breast epithelial cells was controlled by dietary consumption of soy protein. Our studies also revealed that PBRs may play an important role in angiogenesis, and expression of

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some key angiogenic factors, such as b-FGF and VEGF in breast tumors was lower in soy protein group than in casein group. Therefore, it can be suggested that the breast cancer suppressing effect of dietary soy protein is mediated by inhibition of PBRs-mediated angiogenic signaling. It is thus important that this hypothesis is tested by future studies to open-up a new therapeutic approach by control of PBRs-mediated angiogenesis. To accomplish this goal, we have submitted a new Idea proposal entitled "Can Dietary Soy Protein Inhibit Breast Cancer Progression by Control of PBRs-Mediated Angiogenesis" (BC 061674) to the Breast Cancer Research Program of the Department of Army.

<u>Key Research Accomplishment</u>:

1. Development of breast cancer model in rats by dietary feeding of casein as source of protein and administration of DMBA by gavage. Majority of cancers was of aggressive type (60% grade II and 20% grade III). Only 20% was of the grade I (non-aggressive) type.
2. Dietary consumption of soy protein has a beneficial effect. It delays the onset of cancers and also produces a less aggressive cancer (100% non-aggressive). Therefore, prognosis will be better if soy protein is consumed in lieu of casein as dietary source of protein.
3. Even though both casein and soy protein fed animals had breast cancer when gavaged with DMBA, soy protein consumption possibly retards the progression of angiogenesis.
4. We have been successful in isolating and culturing rat mammary epithelial cells.
5. We have established that beneficial effect of soybean protein in delaying the progression of breast cancer is mediated by its down-regulation of the expression of PBRs.
6. This study is the first to report that PBRs play a role in angiogenesis.
7. The beneficial effect of soy protein is probably mediated by control of PBRS-mediated angiogenesis.
8. Dietary consumption of soy protein decreases cell proliferation by inhibition of the expression of AP-1 transcription factors (c-jun and c-fos) and several members of the MAPK family, such as P-38 and JNK-2.

<u>Reportable Outcomes</u>:

Manuscript:

1. Sinha Roy, S., Mukherjee, S., Mukhopadhyay, S., and Das, S. K. Differential Effect of Cadmium on Cholinephosphotransferase Activity in Normal and Cancerous Mammary Epithelial Cell Lines, Mol. Cancer Therapeutics 3(2): 199-204, 2004.
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3. Akech J, Sinha Roy, S. and Das S.K. Modulation of Cholinephosphotransferase Activity in Breast Cancer Cell Lines by Ro5-4864, a Peripheral Benzodiazepine Receptor Agonist. Biochem. Biophys. Res. Commun., 333: 35-41, 2005.
4. Mukhopadhyay S., Ballard B., Mukherjee S. Kabir SM and Das SK. Beneficial Effects of Soy Protein in the Initiation and Progression Against Dimethylbenz[a]anthracene-Induced Breast Tumors in Female Rats. In Press, Mol. Cell. Biochem, 2006
5. Mukhopadhyay S, Mukherjee and Das SK. Increased Expression of Peripheral Benzodiazepine Receptor (PBR) in Dimethylbenz[a]anthracene-Induced Mammary Tumors in Rats. Glycoconjugate J. 23: 201-209, 2006.

Abstract:

1 Mukhopadhyay, S., Das, S. K. and Mukherjee, S. Effects of Cadmium on Antioxidant Defense System in Normal and Malignant Breast Cell Lines. Annual Experimental Biology Meeting, San Diego, California, April 11-15, 2003.

2 Sinha Roy, S., Mukherjee, S., Mukhopadhyay, S. and Das, S. K. Differential Effect of Cadmium on Cholinephosphotransferase Activity in Normal, Non-Aggressive and Aggressive Cancerous Human Mammary Epithelial Cell Lines. Annual Experimental Biology Meeting, San Diego, California, April 11-15, 2003.

3 Akech, J. and Das, S. K. Correlation between Expression of the Peripheral Benzodiazepine Receptors and Breast Cancer Cell Proliferation, IUBMB/ASBMB 2004 Meeting, Boston, MA, June 12-16, 2004.

4 Mukhopadhyay S, Ballard BR, Mukherjee S, Das SK. Potential Role of Peripheral Benzodiazepine Receptor (PBR) Expression as a Biomarker for Breast Cancer. 7th International Symposium on Biochemical Roles of Eukarytic Cell Surface Macromolecules, Jan 10-14, 2005, Puri, India.

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6 SinhaRoy S, Mukhopadhyay S, Mukherjee S and Das SK. Modulation of Expression of Cholinephosphotransferase in Breast Cancer. FASEB J., Vol. 19, A841, 2005, FASEB Meeting, April 2-6, San Diego, CA.

7 Mukhopadhyay S, Ballard BR, Mukherjee S, and Das SK. Increased Expression of Peripheral Benzodiazepine Receptor (PBR) in Dimethylbenz[a]anthracene-Induced Mammary Tumors in Rats. FASEB J., Vol. 19, A280, 2005 FASEB Meeting, April 2-6, San Diego, CA.

8 Mukhopadhyay S, Ballard BR, Mukherjee S, Das SK. Increased Expression of Peripheral Benzodiazepine Receptor (PBR) in Breast Cancer. ERA of Hope 2005 DOD Breast Cancer Research Program Meeting, Philadelphia, PA, June 8-11, 2005.

9 Mukhopadhyay S, Mukherjee S, and Das SK. Beneficial Effects of Dietary Soybean Protein in Prevention of DMBA-Induced Breast Cancer in Rats. XVIII International Congress of Nutrition, Durban, South Africa, Sept 19-23, 2005.

10 Mukhopadhyay S., Ballard B.R., Mukherjee S., Kabir S.M. and Das S. K. Beneficial Effects of Soy Protein in the Development of DMBA-Induced Breast Tumors in Rats., Abstract # 1224, FASEB Meeting, April 1-5, 2006, San Francisco, CA.

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